REMARKS

Claims 6-12, 38-45, 47, and 48 are active in the present application. Support for the amendment to Claim 48 is found on page 6, lines 18-20: "The lineage committed human cell used in accordance with the present invention are cells which are differentiated to at least a point where they are programmed to develop into a specific type of cell." In addition, support for the amendment to claim 48 is found in Claim 42 (prior to the current amendment) and Claim 47. No new matter is believed to have been added by these amendments.

The present invention provides a method for obtaining lineage committed cells, which are those cells that are differentiated to at least a point where they are programmed to develop into a specific type of cell, with enhanced biological function by culturing with a liquid medium replacement rate of at least 25% daily replacement for more than one day (see Claim 38). This method is not described in the prior art cited for the following reasons.

Emerson et al (U.S. Patent No. 5,437,994) describe culturing human bone marrow stromal cells by a method where a liquid culture medium is replaced or perfused at a specified rate (see col. 4, lines 39 through col. 5, line 9 and col. 7, lines 60-64). However, human bone marrow stromal cells are not lineage committed cells as defined in the present claims, i.e., "differentiated to at least a point where they are programmed to develop into a specific type of cell." Bone marrow stromal cells are known to be multipotent or cells that can develop into many different types of cells, i.e., progenitor or stem cells, which is supported by the general knowledge concerning bone marrow stromal cells (see the attached publication: Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* 1997 Apr 4;276(5309):71-4).

In view of the above, Applicants request withdrawal of the rejection over Emerson et al.

Similarly, Caldwell et al describe culturing bone marrow stromal cells to detect GM-CSF secretion (see page 350, column 1). As noted above, bone marrow stromal cells are NOT lineage committed human cells as in the present claims. Therefore, the present claims cannot be anticipated by Caldwell et al and withdrawal of the rejection over Caldwell is requested.

Turning to the rejection over Freedman et al, Applicants point out that the method of Freedman et al involves culturing tumor infiltrating lymphocytes with the following steps: "The cultures were typically inspected every 3-4 days and fed by removing one half of the medium in the wells and replacing it with fresh complete AIM V medium." (See the paragraph bridging pages 147-148). The Freeman et al method also includes transferring cells to a flask that "were fed with fresh complete AIM V medium twice weekly" (page 148, col. 1, paragraph 1); adding fresh medium to maintain cell concentration where "[c]ell numbers and viability were determined every 3-4 days" (page 148, col. 1 paragraph 2); and then through a artificial capillary culture system to circulate the media for "oxygenation and gas exchange" (page 148, col. 1, paragraph 3).

However, Freedman et al do not describe the claimed method of culturing a lineage committed human hematopoietic cell composition which includes a liquid medium replacement rate of at least 25% daily replacement for more than one day (see Claim 38). Therefore, the present claims cannot be anticipated by Freedman et al and therefore, withdrawal of this ground of rejection is requested.

The objections of Claim 9 and 43 are addressed by amendment.

The rejection of Claims 7-12, 38-45 and 47-48 under 35 U.S.C. § 112, second paragraph is addressed by amendment. Claim 38 is amended to clarify that the lineage committed cells are those cells that are differentiated to at least a point where they are programmed to develop into a specific type of cell (see also page 6, lines 18-20).

The rejection of Claims 7-12, 38-45 and 47 under 35 U.S.C. § 112, first paragraph is addressed by amendment. Claim 38 is amended to clarify that the lineage committed cells cultured are lineage committed hematopoietic cells, which is described and enabled by the specification on page 7, lines 14-18 and lines 20-22); pages 12-19; and the Examples. Therefore, withdrawal of this rejection is requested.

In the event the Examiner has any questions or requires clarification on any issue in this application, he is invited to contact the Applicants' undersigned representative to resolve the matter expediently.

Applicants submit the present application is now ready for allowance. Early notification of such allowance is earnestly solicited.

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Marrow Stromal Cells as Stem Cells for Nonhematopoietic Tissues

Darwin J. Prockop

Marrow stromal cells can be isolated from other cells in marrow by their tendency to adhere to tissue culture plastic. The cells have many of the characteristics of stem cells for tissues that can roughly be defined as mesenchymal, because they can be differentiated in culture into osteoblasts, chondrocytes, adipocytes, and even myoblasts. Therefore, marrow stromal cells present an intriguing model for examining the differentiation of stem cells. Also, they have several characteristics that make them potentially useful for cell and gene therapy.

Because circulating blood cells survive for only a few days or months, hematopoietic stem cells (HSCs) in bone marrow must provide a continuous source of progenitors for red cells, platelets, monocytes, granulocytes, and lymphocytes (*1*). However, bone marrow also contains cells that meet the criteria for stem cells of nonhematopoietic tissues. The stem-like cells for nonhematopoietic tissues are currently referred to either as mesenchymal stem cells, because of their ability to differentiate into cells that can roughly be defined as mesenchymal, or as marrow stromal cells (MSCs), because they appear to arise from the complex array of supporting structures found in marrow.

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Multipotentiality of MSCs

The presence of stem cells for nonhematopoietic cells in bone marrow was first suggested by the observations of the German pathologist Cohnheim 130 years ago (*2*). Cohnheim studied wound repair by injecting an insoluble analine dye into the veins of animals and then looking for the appearance of dye-containing cells in wounds he

created at a distal site. He concluded that most, if not all, of the cells appearing in the wounds came from the bloodstream, and, by implication, from bone marrow. The stained cells included not only inflammatory cells but also cells that had a fibroblast-like morphology and were associated with thin fibrils. Therefore, Cohnheim's work raised the possibility that bone marrow may be the source of fibroblasts that deposit collagen fibers as part of the normal process of wound repair. The source of fibroblasts in wound repair has been examined in more than 40 publications since Cohnheim's report of 1867 (3, 4). Most of the data suggest that the fibroblasts are of local origin, but the issue has not been resolved and is still being examined (4).

Although Cohnheim's thesis has not yet been substantiated, definitive evidence that bone marrow contains cells that can differentiate into fibroblasts as well as other mesenchymal cells has been available since the pioneering work of Friedenstein, beginning in the mid-1970s (5). Friedenstein placed samples of whole bone marrow in plastic culture dishes, and, after 4 hours or so, poured off the cells that were nonadherent. In effect, he discarded most of the HSCs and their hematopoietic progeny that are of interest in the field of bone marrow transplantation. He reported that the small number of adherent cells were heterogeneous in appearance, but the most tightly adherent cells were spindle-shaped and formed foci of two to four cells. The cells in the foci remained dormant for 2 to 4 days and then began to multiply rapidly. After passage several times in culture, the adherent cells became more uniformly spindle-shaped in appearance. The most striking feature of the cells, however, was that they had the ability to differentiate into colonies that resembled small deposits of bone or cartilage.

Friedenstein's initial observations were extended by a number of investigators during the 1980s, particularly by Piersma and associates (6) and by Owen and associates (7). These and other studies (8–11) established that the MSCs isolated by the relatively crude procedure of Friedenstein were multipotential and readily differentiated into osteoblasts, chondroblasts, adipocytes, and even myoblasts (9). Most impressively, Friedenstein *et al.* (10) demonstrated that even after 20 or 30 cell doublings in culture, MSCs still differentiated into fibrous tissue, bone, and some cartilage when enclosed in a capsule with a porous membrane and implanted into the peritoneum of rats.

Even though the multipotential properties of MSCs have been recognized for several decades, there are surprisingly large gaps in our information about the cells themselves. The cells, isolated by their adherence to plastic as described by Friedenstein (5), initially are heterogeneous and are difficult to clone. The fraction of hematopoietic cells is relatively high in initial cultures of mouse marrow but is less than 30% with human marrow (8, 11). Most of the readily identifiable hematopoietic cells are lost as the cells are maintained as primary cultures for 2 or 3 weeks. The cultured MSCs synthesize an extracellular matrix that includes interstitial type I collagen, fibronectin, and the type IV collagen and laminin of basement membranes (8, 11). A small fraction of the cultured cells synthesize factor VIII–associated antigen and therefore are probably endothelial. The cells secrete cytokines, the most important of which appear to be interleukin-7 (IL-7), IL-8, IL-11, and stem cell factor (c-kit ligand). Conditions for differentiating the cells are somewhat species-dependent and are influenced by incompletely defined variables, such as the lot of fetal calf serum used. However, MSCs from mouse, rat, rabbit, and human readily differentiate into colonies of osteoblasts (depositing mineral in the form of hydroxyapatite), chondrocytes (synthesizing cartilage matrix), and adipocytes in response to dexamethasone, 1,25-dihydroxyvitamin D_3, or cytokines such as BMP-2 (5–11). In response to 5-azacytidine and amphotericin B or amphotericin B alone (9), they differentiate into myoblasts that fuse into rhythmically beating myotubes.

Most experiments on the differentiation of MSCs have been carried out with cultures of MSCs as described by Friedenstein (5), but several groups of investigators since 1990 have attempted to prepare more homogeneous populations (12–16). The protocols developed by these investigators for the isolation of MSCs have several advantages: The isolated cells are either clonal or nearly clonal, they express small amounts of bone cell markers such as alkaline phosphatase, and they can be induced in culture to express large amounts of the same markers and to form mineralizing colonies. None of the protocols, however, has yet been used in more than one laboratory, and it has not been shown whether they isolate the same cells. Also, although the cells isolated with several of the protocols differentiate into osteoblasts in culture, it has not been demonstrated that they retain all the multipotential properties of MSCs isolated by Friedenstein's protocol, such as the potential for differentiating into adipocytes, chondrocytes, and myotubes (5–10).

Among the largest gaps in our information is whether MSCs isolated by their adherence to plastic in the absence of nonadherent hematopoietic cells differ from the apparently similar cells that are used as feeder layers for long-term cultures of HSCs (17–19). Early in the study of HSCs, it was recognized that when samples of whole marrow are placed in culture dishes, the small number of cells that adhere to the plastic provide an important microenvironment for both the growth of HSCs and their differentiation into granulocytes and erythrocytes (17). In long-term cultures of HSCs, the adherent cells interact directly with hematopoietic precursors. In some discrete regions of attached cells in culture, extremely large cells with a thin cytoplasm form blankets over granulocyte precursors, resulting in a cobblestone appearance. As was demonstrated by time-lapse photography, the granulocyte precursors in the medium move under the blanket cells, replicate, differentiate, and then move out into the medium as they mature. Other regions of the same cultures form isolated clusters of macrophages and erythroblasts in which the erythroblasts undergo synchronous maturation and enucleation. The adherent cells in the cultures provide secreted cytokines that include IL-1, IL-6, colony-stimulating factor–1 (CSF-1), granulocyte-macrophage-CSF, macrophage-CSF, and c-kit ligand (18). They also provide matrix-bound cytokines and important but still undefined cell-cell contacts (11, 17, 18). The cultures of HSCs can be maintained for 20 weeks or more, and the HSCs recovered from long-term cultures readily differentiate into mature blood cells in response to a series of well-defined cytokines (11, 17). The adherent cells used as feeder layers for HSCs have many of the characteristics of MSCs isolated by their adherence to plastic in the absence of nonadherent cells, but it is not clear whether they retain the potential to differentiate into bone, cartilage, and other mesenchymal cells, or whether they have differentiated into another and discrete phenotype because of their continuing interaction with hematopoietic cells.

Another gap in the information about MSCs concerns the precise molecular events involved in their differentiation. In a mixed population of HSCs and related progenitors that are $CD34^+$, at least 13 known *HOX* genes and five novel homeodomain-containing genes are expressed (20). However, the sequence of expression of these and related developmental genes has not been defined. Also, the expression of such developmental genes has not been examined in MSCs themselves. Nor has it been established whether MSCs differentiate directly into osteoblasts, chondrocytes, and adipocytes, or whether one or more of the cells are on the same pathway of differentiation.

Why Does Marrow Contain MSCs?

Why does marrow contain cells with the potential to differentiate into a variety of mesenchymal cells? The differentiation of MSCs into bone is not in itself surprising. Marrow, particularly in humans, contains a complex array of thin spicules of trabecular bone, which is similar to other bone in that it continually undergoes remodeling (*21*). Therefore, it is not surprising that samples of marrow extruded from bone include osteoblast precursors that may have been eluted from trabecular bone or the inner surface of the bone itself. The potential of MSCs to differentiate into adipocytes may be related to the observation that marrow is partially replaced by adipose tissue with aging. Also, some forms of osteoporosis may be caused by an increased tendency of osteoblasts or osteoblast precursors in bone to differentiate into adipocytes (*22*). The potential of MSCs to differentiate into chondrocytes may be related to the process of fracture repair, because small amounts of cartilage frequently appear at fracture sites as the initially formed callus is replaced by bone. However, fracture repair readily occurs in bone that lacks marrow, and it has been generally assumed that the reparative cells arise from the fracture site itself (*21*). The potential of MSCs to differentiate into myoblasts and even myotubes (*11*) is even more difficult to explain, because muscle cells cannot be replaced after they are destroyed in adults. Therefore, the differentiation into myoblasts in culture may reflect a multipotential feature of the cells that is not realized in vivo.

We in our laboratory (*23*) tried to explore the question of why marrow contains multipotential MSCs by addressing a simpler experimental question: Where do MSCs and the progeny of MSCs go after systemic infusion? Earlier experiments (*24*) demonstrated that infused MSCs repopulate up to one-third of the MSCs in the marrow of recipient animals that have undergone marrow ablation to create a space for engraftment of cells. In our own experiments, we were primarily interested in whether the infused MSCs or their progeny repopulated nonhematopoietic cells and tissues. Therefore, we used MSCs, prepared as described by Friedenstein and others (*5–10*), from a line of transgenic mice expressing a mutated collagen gene (*25*). The mutated gene for type I collagen was expressed in a tissue-specific manner and served as a marker both for the presence of the donor MSCs and for the tissue-specific expression of any cells that contained the marker gene. The marked MSCs were infused into isogenic mice that were x-ray irradiated (*23*). After 1 week, a sensitive polymerase chain reaction (PCR) assay that could detect about one donor cell per 10,000 cells was unable to detect the donor MSCs in any tissues of the recipient mice (Fig. 1). At 1 and 5 months, however, the donor cells accounted for 1 to 12% of the cells in a number of tissues. The extent of cell replacement was essentially the same in bone and cartilage as in marrow and spleen. Also, the marker gene was found in cells that were cultured from pieces of bone and passed three times (*23*). Moreover, expression of the mutated collagen gene as messenger RNA was detected in the cultured bone cells. In contrast, cells containing the mutated collagen gene were present in cartilage from the recipient mice, but the mutated gene was not expressed. Because the marker gene was for type I collagen, a gene that is expressed in bone but not in cartilage (*25*), the results suggested that the progeny of MSCs expressed genes in a tissue-specific manner. In similar experiments, Keating *et al.* (*26*) detected either donor MSCs or their progeny in liver, thymus, and lung as well as in marrow and spleen after infusion of human MSCs into nonirradiated SCID (severe combined immunodeficiency disease) mice. At 2 months, the donor cells accounted for 0.2 to 2.3% of the cells in liver, thymus, and lung.

Our observations (*23*) and those of Keating *et al.* (*26*) suggested that the donor MSCs first replace a portion of the MSCs in the bone marrow of the recipient mouse. The MSCs then participate in a normal biological cycle in which MSCs in the bone marrow serve as a continuing source of progenitor cells for a variety of mesenchymal tissues. Moreover, the expression of the marker gene for type I collagen in bone but not in cartilage (*23*) suggested that the progeny of MSCs acquire the phenotypes of different target tissues either before they leave the marrow or after they have entered the microenvironment of the tissue itself. These results did not establish that MSCs are the only source of progenitor cells for mesenchymal tissues, but they demonstrated that MSCs can make important contributions. Also, although the results did not directly address the source of fibroblasts in wound healing, they obviously were consistent with Cohnheim's original thesis (*2*).

Potential Uses in Cell and Gene Therapy

Within the past several years, MSCs have been explored as vehicles for both cell therapy and gene therapy. The cells are relatively easy to isolate from the small aspirates of bone marrow that can be obtained under local anesthesia; they are also relatively easy to expand in culture and to transfect with exogenous genes (*11*, *24*, *26*). Therefore, MSCs appear to have several advantages over HSCs for use in gene therapy. The isolation of adequate numbers of HSCs requires large volumes of marrow (1 liter or more), and the cells are difficult

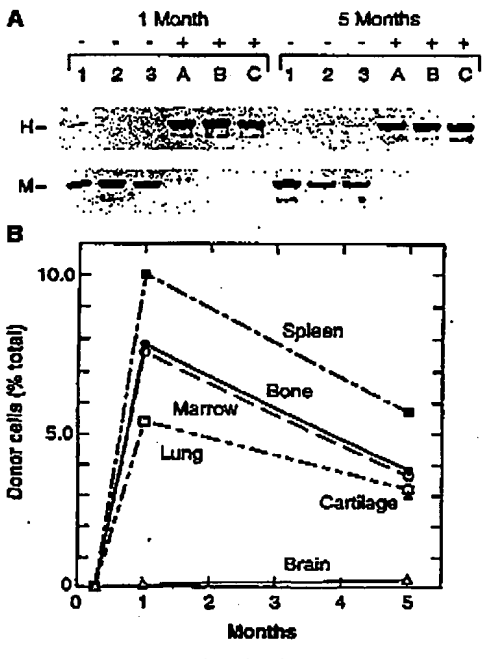

Fig. 1. Distribution of progeny of donor MSCs after infusion into x-ray–irradiated mice (*23*). Recipients were 8- to 10-week-old mice from an inbred strain (FVB/N) that each received potentially lethal x-ray irradiation (9.0 Gy) before intravenous infusion of 1×10^6 cultured MSCs from a transgenic mouse with a mutated human *COL1A1* gene together with 6×10^5 freshly isolated nonadherent cells from a normal mouse from the same strain as a source of hematopoietic cells. The mutated human *COL1A1* gene and the endogenous mouse *Col1a1* gene were assayed with a PCR assay that used the same two primers for both genes but generated a smaller fragment from the mouse *Col1a1* gene because of a natural deletion in the 5′-nontranslated region. After 1 week, the marker human *COL1A1* gene was not detected in tissues from the recipient mice even though the assay would have detected one donor cell per 10,000 cells. At 1 month and 5 months, progeny of the donor MSCs accounted for 1 to 12% of the cells in marrow, spleen, bone, lung, and cartilage. Because of the relatively large variation in the data, the values at 1 month and 5 months are not significantly different. (A) Assay of ^{32}P-labeled PCR products obtained with DNA isolation from bone as a template and separated on a 7% polyacrylamide–6 M urea gel. The gel was assayed with a phosphostimulatable storage plat (PhosphorImager, Molecular Dynamics). (B) Values for several tissues expressed as percent of donor MSCs or progeny of donor MSCs per total cells. Symbols: + and −, mice that did or did not receive x-ray irradiation before infusion of donor MSCs plus nonadherent cells; 1 to 3 and A to C, arbitrary notations assigned to mice killed at 1 month or 5 months.

to expand in culture (*17*, *27*).

Several different strategies are being pursued for the therapeutic use of MSCs. A strategy first proposed by Caplan and coworkers (*28*) is to isolate MSCs from the bone marrow of a patient with degenerative arthritis, expand the MSCs in culture, and then use the cells for resurfacing of joint surfaces by direct injection into the joints. Alternatively, the MSCs can be implanted into poorly healing bone to enhance the repair process. Locally injected MSCs were shown to promote repair of surgical incisions in the knee cartilage of rabbits, and MSCs in ceramic beads were shown to promote bone healing in an animal model (*28*). Several attempts have been made to use chondrocytes to resurface joint cartilage or in reconstructive plastic surgery in patients with osteoarthritis (*29*), but the supply of normal chondrocytes from patients is severely limited. Therefore, MSCs that can differentiate into chondrocytes are an attractive alternate source.

A second strategy for the use of MSCs is to introduce genes for secreted proteins into the MSCs and then infuse the cells systemically so that they return to the marrow and secrete the therapeutic protein. Alternatively, the MSCs secreting a therapeutic protein can be encapsulated in some inert material that allows diffusion of proteins but not the cells themselves. Keating *et al.* (*26*) demonstrated that human MSCs transfected with a gene for factor IX secrete the protein for at least 8 weeks after systemic infusion into SCID mice. Therefore, gene-engineered MSCs may be an effective vehicle for therapy of hemophilia B and other genetic diseases caused by deficiencies in circulating proteins.

A third strategy is to infuse MSCs systemically under conditions in which the cells will not only repopulate bone marrow, but also provide progeny for the repopulation of other tissues such as bone, lung, and perhaps cartilage and brain. In recent experiments, we found that when donor MSCs from normal mice are infused in large amounts into young mice that are enfeebled because they express a mutated collagen gene, the normal donor cells replace up to 30% of the cells in bone, cartilage, and brain of the recipient mice (*23*). These results were the basis of a clinical trial now in progress (*30*) for the therapy of bone defects seen in children with severe osteogenesis imperfecta caused by mutations in the genes for type I collagen. The children undergo marrow ablation followed by transplantation of normal marrow from a human leukocyte antigen (HLA)–matched donor. The trial is based on the hypothesis that whole bone marrow may contain enough MSCs to replace a sufficient number of osteoblasts to convert a severe bone defect into a relatively mild one. One possible strategy for the future is to isolate MSCs from a patient with severe osteogenesis imperfecta, replace the mutated gene for type I collagen by homologous recombination in culture, and then return the cells to the patient. A phase I clinical trial demonstrated that the systemic infusion of autologous MSCs appears to be well tolerated (*31*). Also, several reports suggested that engraftment of whole marrow or of MSCs can be obtained in mice or dogs without the need for marrow ablation (*26*, *32*) if large numbers of cells are infused or if they are infused at regularly spaced intervals (*32*). Therefore, it may be possible to use gene-engineered MSCs from a patient for therapy of common diseases, such as osteoporosis, in which marrow ablation cannot be justified. Obviously, however, a number of fundamental questions about MSCs still need to be resolved before they can be used for safe and effective cell and gene therapy.

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